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Biosynthesis and Structure Activity Relationship Investigations of the Diazeniumdiolate Antifungal Agent Fragin

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Only a few natural products possessing a diazeniumdiolate have been isolated and usually these compounds display a broad range of biological activities. Only recently the first diazeniumdiolate natural product biosynthetic gene cluster was identified in *Burkholderia cenocepacia* H111, which produces the fungicide (–)-fragin and the signal molecule (*rac*)-valdiazen. In this study, L-valine was identified as the initial substrate of (–)-fragin biosynthesis by feeding experiments using isotopically labeled amino acids. The formation of the diazeniumdiolate was chemically studied by several proposed intermediates. Our results indicate that the functional group is formed during an early stage of the biosynthesis. Furthermore, an oxime compound was identified as a degradation product of (–)-fragin and was also observed in the crude extract of the wild type strain. Moreover, a structure-activity relationship analysis revealed that each moiety of (–)-fragin is essential for its biological activity.

The diazeniumdiolate moiety is a fascinating functional group containing two O- and two N-atoms,[1,2] and compounds with this group have been discovered already more than two centuries ago.[3] In 1966, the first natural product possessing a diazeniumdiolate group was isolated from *Streptomyces alanosinicus* and named alanosine.[4] This compound displayed strong antiviral and antitumor activity, and reached a phase II clinical study for its potent antimetabolite activity.[5] Since this discovery, only a few new diazeniumdiolate natural products have been identified,[6] such as *e.g.* (–)-fragin (**1**), which was isolated from the culture supernatant of *Pseudomonas fragi*. This compound was shown to possess antifungal, antitumor, antibacterial, and plant growth inhibitory activities.[7-11] More recently, the novel signaling molecule (*rac*)-valdiazen (**2**) was isolated from *B. cenocepacia* H111 (H111) using a metagenomic approach,[12-14] and Hertweck and co-workers discovered in the same year a new family of diazeniumdiolate siderophores[15,16]. The discovery of (*rac*)-valdiazen (**2**) was initiated by the identification of the (–)-fragin (**1**) gene cluster, which was the first biosynthetic gene cluster encoding for a diazeniumdiolate compound. The (–)-fragin gene cluster consists of seven genes separated in two oppositely orientated operons (figure **1**). The predicted functions of the corresponding proteins include a haem-oxygenase for HamA, a cupin domain superfamily protein for HamB, a *p*-aminobenzoate *N*-oxygenase for HamC, a non-ribosomal peptide synthetase (NRPS)-like protein for HamD, a polyketide cyclase/dehydratase for HamE, a starter condensation domain for HamF, and an aminotransferase for HamG.[13] Based on the structure of (*rac*)-valdiazen (**2**) and (–)-fragin (**1**), L-valine (**3**) was hypothesized to be the substrate for their biosyntheses. Our suggestion is in agreement with a recent study performed on PvfC,[17] a homolog of HamD initially discovered in a *Pseudomonas entomophila* strain.[18] Li and coworkers identified the key residues of the active side, and demonstrated by bioinformatics and experimental studies that L-valine (**3**) is the initial substrate of the PvfC NRPS. Furthermore, our group investigated the *p*-aminobenzoate *N*-oxygenase enzyme HamC, a homologue of AurF.[19] HamC was produced, isolated and, similar to AurF[19], shown to oxidize *p*-aminobenzoic acid into *p*-nitrobenzoic acid.[13] Furthermore, preliminary experiments explored the origin of the terminal N atom of the diazeniumdiolate group and chromatographic evidence suggested it originated from nitrite. Recently, several reports indicated similar biosynthetic pathways[20,21] for the diazeniumdiolate alanosine,[22] the diazo compounds cremeomycin[23-26] and kanamycin,[27] the pyridazine azamerone,[28] the *N*-hydroxytriazene,[29] and the hydrazide fosfazinomycin.[27,30]

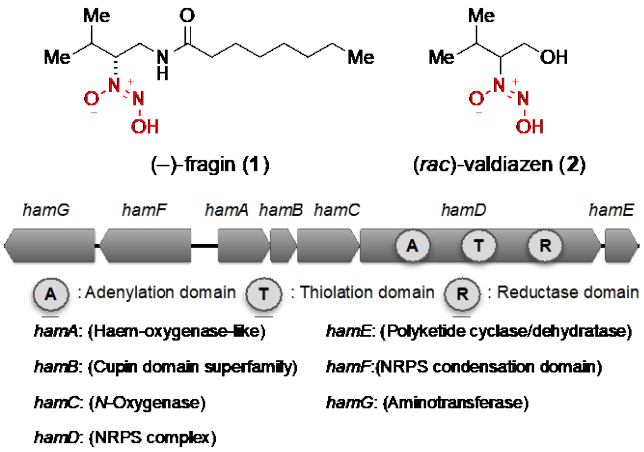

Figure 1. Constitution and configuration of the natural products (–)-fragin (**1**) and (*rac*)-valdiazen (**2**), and their biosynthetic gene cluster identified in the *B. cenocepacia* H111 genome. The diazeniumdiolate function group is highlighted in red and the predicted function of the genes is indicated in brackets.

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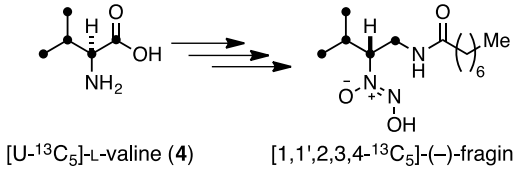
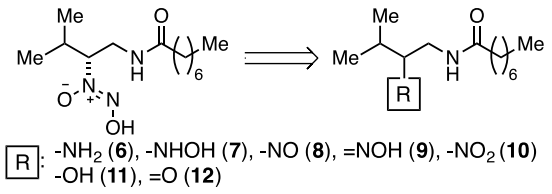
In this study, we continued our biosynthetic investigations of (–)-fragin (**1**) with the determination of the initial substrate of the HamD NRPS and with the search for biosynthetic intermediates. Furthermore, we hypothesized that the biological activities of (–)-fragin (**1**) required all chemical features present in the natural product. Several derivatives were designed, synthesized, and tested in order to experimentally corroborate these hypotheses.

Our biosynthetic investigations started with the identification of the amino acid substrate of the NRPS complex HamD. *In silico* analysis of the HamD adenylation domain predicted phenylalanine (NRPSsp[31]) or leucine (antiSMASH 3.0[32] and NRPSpredictor 2[33]) to be the amino acid substrate. However, based on the structure of fragin, we proposed that either valine or leucine is the substrate of the HamD adenylation domain. This is supported by recently published data that identified valine as the amino acid substrate of PvfC, which has an identical 10 amino acid code in its adenylation domain to HamD.[17] To test valine incorporation into fragin, feeding experiments were performed using [U-$^{13}C_5$]-L-valine (**4**) and the supernatant was analysed by HRMS. The pseudomolecular ion $[M+Na]^+$ with an *m/z* of 301.2108 Da was detected, indicating the incorporation of five labelled C atoms into (–)-fragin (**1**). Relative quantification showed that 9.7 % of total fragin from this feeding experiment was isotopically labelled fragin (Scheme **1**) suggesting that L-valine (**3**) is the initial substrate for HamD.



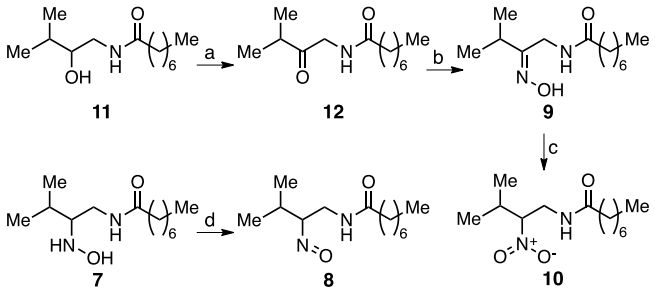
Scheme 1. Feeding experiment with [U-$^{13}C_5$]-L-valine (**4**) demonstrated the incorporation of [U-$^{13}C_5$]-L-valine (**4**) into fragin [1,1,2,3,4-$^{13}C_5$]-fragin (**5**).

We continued our investigation by identifying at which biosynthetic step the diazeniumdiolate group is formed. Due to the instability of this functional group, we first hypothesized that it could be formed late in the biosynthesis. Several possible intermediates were proposed, including different oxidation states of the amino or hydroxy group such as the amine **6**, hydroxylamine **7**, nitroso compound **8**, oxime **9**, nitro compound **10**, hydroxy compound **11** and keto derivative **12** (Scheme **2**).



Scheme 2. (–)-Fragin (**1**) biosynthesis proposal considering a late stage diazeniumdiolate formation.

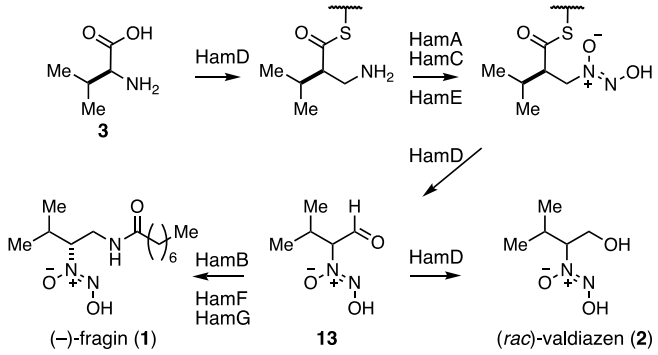
The amine **6** and hydroxylamine **7** derivatives were synthesized following a recently reported procedure.[13] Peptide coupling was performed with 1-amino-3-methylbutan-2-ol and octanoic acid using HATU to obtain the hydroxy compound **11**, which was oxidized with the Dess-Martin periodinane (DMP) reagent into the ketone **12** and further reacted with NH_2OH x HCl to obtain the oxime **9**. An additional oxidation of the oxime **9** with meta-chloroperoxybenzoic acid (*m*CPBA) led to the nitro compound **10** and the oxidation of the hydroxylamine **7** gave the nitroso derivative **8** (Scheme **3**).



Scheme 3. Synthesis of biosynthetic proposed intermediates. a) DMP, CH_2Cl_2, 4 h, 46%; b) NH_2OH x HCl, NaOAc, MeOH, 85°C, 8 h, 44%; c) Urea, Na_2HPO_4, MeCN, reflux, 30 min, *m*-CPBA, reflux, 2 h, 54%; d) *m*-CPBA, $CHCl_3$, 0°C, 2 h, RT, 1 h, 99%.

The supernatants of the wild type and the previously reported mutant strains Δ*hamA*, Δ*hamB*, Δ*hamC*, Δ*hamD*, Δ*hamE*, Δ*hamF*, and Δ*hamG*[13] were extracted with $CHCl_3$ and analyzed by HPLC-MS using the synthesized derivatives as analytical standards. Interestingly, the oxime **9** was present in small amounts in the extract of the wild type strain (Supporting info), which however could also be formed during the decomposition of fragin.[10] However, none of the derivatives were detected in the extracts of the mutants. These results including the discovery and isolation of (*rac*)-valdiazen (**2**) suggested that the formation of the diazeniumdiolate occurred during an early step of the biosynthetic pathway. We proposed that L-valine (**3**) would be the initial substrate of the NRPS complex HamD and that the enzymes of unknown functions, HamA and HamE, and the *N*-oxygenase analog HamC would transform the amine into the diazeniumdiolate. This hypothesis is supported by the previous identification of several modifications occurring during chain elongation[34-36] and by recent investigations on alanosine biosynthesis.[22,37] The compound would be released as an aldehyde by the reductase domain of HamD and further reduced by the same enzyme to obtain (*rac*)-valdiazen (**2**). A similar biosynthetic pathway was proposed for the formation of myxochelins A and B involving a NRPS reductase domain performing two and four-electron reductions.[38] Furthermore, the presence of a biosynthetic intermediate that racemized was hypothesized due to the inverse configuration of the stereogenic center of (–)-fragin (**1**), when compared to its initial substrate L-valine (**3**) and the isolation of (*rac*)-valdiazen (**2**) as a racemate. The possible intermediate **13** was proposed to racemize due to the presence of a reactive aldehyde, at the beta position of the diazeniumdiolate, which could interconvert to its enolate tautomer form. Finally, HamB, the aminotransferase HamG, and the starter condensation domain protein HamF would be involved in the last steps of of (–)-fragin biosynthesis (**1**). This is supported by the fact

that these enzymes were not essential for the production of (*rac*)-valdiazen (**2**) (Scheme **4**).[13]



Scheme 4. Biosynthesis proposal of the natural product (–)-fragin (**1**) and (*rac*)-valdiazen (**2**) including the racemization via the aldehyde intermediate **13**.

Intrigued by the presence of the oxime **9** in the wild type strain, we decided to investigate the formation of the diazeniumdiolate on a model substrate. As it was hypothesized before, the diazeniumdiolate could be formed by reacting a hydroxylamine with an NO donor source. We treated the hydroxylamine **7** with sodium nitrite in an aq. methanol solution ensuring the solubility of the reagents and the reaction was analysed by HPLC-MS at the time of addition, after 3.5 h and after 7.6 h. The compounds were identified based on their mass and retention time using the internal standards synthesized above. Directly after the addition of the hydroxylamine **7** all the derivatives were already observed except for the nitro compound **10**, which supported the notion that the formation of the diazeniumdiolate is a fast process. A decrease in the concentration of the hydroxylamine **7** and the nitroso compound **8**, and a constant increase in the concentration of the oxime **9** and the nitro compound **10** would suggest that **10** and **9** would constitute the final and more stable compounds. The concentration of (*rac*)-fragin (**14**) was first increased at the 3.5 h time point but then had dropped after 7.6 h, which was accompanied by an increase in the concentration of the oxime **9**. Taking these results into account, we hypothesized that the hydroxylamine **7** is converted to the diazeniumdiolate (*rac*)-fragin (**14**) that decomposed into the oxime **9** and the nitro compound **10** (Figure **2**). Under these conditions, the major decomposition product detected was the oxime **9**, which was also observed in the crude extract of the *B. cenocepacia* H111 wild type strain and was previously identified as a degradation product of fragin.[10] Using our model system, we hypothesize that the oxime **9** observed in the wild type strain is a degradation product of fragin and not a biosynthetic intermediate.

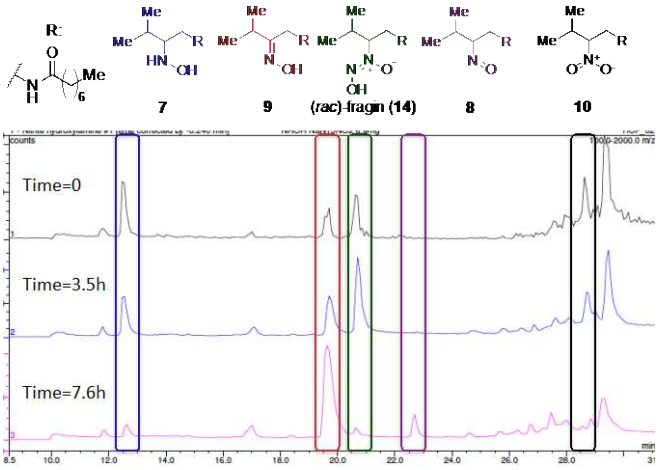


Figure 2. Chromatogram overlays of the reaction between the hydroxylamine and $NaNO_2$ after the addition of the salt, after 3.5 h and after 7.6 h.

In our previous study, (–)-fragin (**1**) was found to possess antifungal activity as well as antibacterial activity against Gram-positive bacteria.[13] A structure-activity relationship (SAR) study was designed to determine which part for the compound is important for its potency and how alterations in its structure might affect the antibacterial and antifungal activity. (–)-Fragin (**1**) is composed of an acyl side chain and an amino acid part originating from L-valine (**3**) and possesses the diazeniumdiolate functional group. Our SAR strategy was designed to independently modify the two different parts of the compound and to evaluate the effect of the diazeniumdiolate functional group.

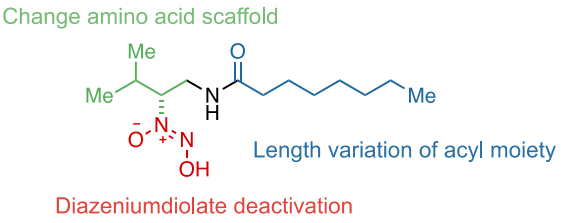
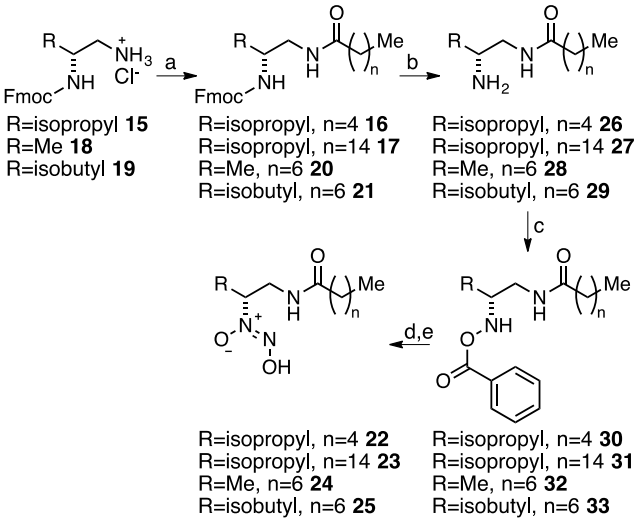


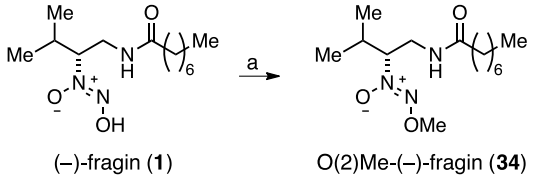
Figure 3. SAR strategy on the modification of (–)-fragin (**1**). The green part highlights the amino acid part, the blue part the acyl moiety and the red part the diazeniumdiolate functional group.

The effect on the acyl moiety length was first investigated with the synthesis of two derivatives possessing acyl side chains composed of 6 or 16 carbons. Furthermore, the amino acids alanine and leucine were selected as a smaller and larger aliphatic residue for the amino acid replacements for fragin. The previously synthesized ammonium salt **15** was coupled to the activated hexanoic acid for **16** and with hexadecanoic chloride for **17,** and the salts **18** and **19** were coupled to octanoyl chloride for **20** and **21**. The Fmoc of the compounds were deprotected, the amine was oxidized and a final treatment with isopentyl nitrite afforded the fragin derivatives **22**, **23, 24** and **25** (Scheme **5**).



Scheme 5. Synthesis of fragin derivatives **22**, **23**, **24** and **25**. a) hexanoyl chloride for **16**, hexadecanoyl chloride for **17** and octanoyl chloride for **20** and **21** DIPEA, DMAP, RT, 14 h, 92% for **16** 75% for **17**, 70% for **20**; b) $AlCl_3$, toluene, RT, 3.5 h 80% for **26**, 45% for **27**, 96% for **28** and 25% over 2 steps for **29**; c) dibenzoylperoxide, K_2HPO_4, THF, RT, 21 h, 77% for **30**, 43% for **31**, 16% for **32** and 42% for **33**; d) N_2H_4 x H_2O, EtOH, RT, 2.5 h; d) isopentyl nitrite, $NH_{3(g)}$, EtOH, RT, 0.5 h, 31% over 2 steps for **22**, 52% over 2 steps for **23**, 65% over 2 steps for **24** and 38% over 2 steps form **25**. Abbreviation: DIPEA: *N,N*-diisopropylethylamine, DMAP: 4-dimethylaminopyridine and THF; tetrahydrofuran.

(–)-Fragin (**1**) was methylated using dimethyl sulfate and sodium carbonate as a base to obtain O(2)Me-(–)-fragin (**34**). As reported previously, the oxygen at the O(2) position of the diazeniumdiolate was protected and gave a stable product (Scheme **6**).[39]



Scheme 6. Synthesis of O(2)Me-(–)-fragin (**34**). a) Na_2CO_3, Me_2SO_4, MeOH, 0°C, 15 min, RT, 2.5 h, 71%.

Inhibition of Gram-negative bacteria could only be observed for (–)-fragin (**1**) with the highest tested concentration (128 µg/ml) against *E. coli*, but not against our two other model organisms, *Pseudomonas putida* and *Klebsiella oxytoca*. A shorter acyl side chain with the derivative **22** was found to lower the activity against the Gram-positive bacteria but to retain the inhibition of *E. coli*. The inverse observation was made for the longer acyl side chain **23** which retained the inhibition of Gram-positive bacteria but showed no more inhibition of *E. coli*. Interestingly, the shorter as well as the longer acyl side chain derivatives (**22** and **23** respectively) showed diminished or no antifungal activity (Figure **4**). The variation of the amino acid moiety with the derivatives **24** and **25** showed the importance of the branched alkyl group for the activity against *B*acillus *cereus* and *Staphylococcus aureus*. Antifungal activity was clearly diminished with the alanine derivative **24** while the leucine derivative **25** displayed only a weak reduction of the antifungal activity compared to **1**. Finally, the methylated diazeniumdiolate (–)-fragin **34** did not display any antibacterial or antifungal activities highlighting the crucial role that this functional group plays in the bioactivity of fragin (Table **1** and Figure **4**).

Table 1 Minimal inhibitory concentration of fragin and its derivatives against Gram-positive (*B. cereus* and *S. aureus*) and Gram-negative (*P. putida*, *E. coli* and *K. oxytoca*) bacteria.

	B. cereus	*S. aureus*	*P. putida*	*E. coli*	*K. oxytoca*
1	**16**	**32**	>128	128	>128
22	128	>128	>128	128	>128
23	**32**	**32**	>128	>128	>128
24	64	128	>128	128	>128
25	**16**	**32**	>128	128	>128
34	>128	>128	>128	>128	>128

The activities are reported in µg/mL. The experiments were performed in triplicate. The activities equal or lower than 32 µg/mL are highlighted in bold and in red. The compounds tested were (–)-fragin (**1**), the shorter acyl side chain **22**, the longer acyl side chain **23**, the alanine **24**, the leucine **25** derivatives, and the methyl protected (–)-fragin **34**.

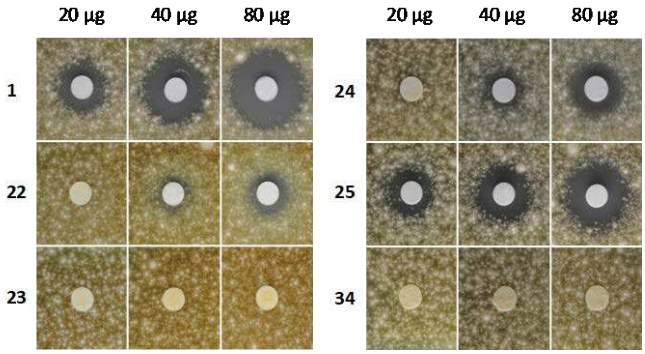


Figure 4. Disc diffusion assays of (–)-fragin (**1**) and its derivatives **22**, **23**, **24**, **25** and **34** to determine their antifungal activity. Three different amounts (20 µg, 40 µg and 80 µg) of each compound were tested. Representative pictures of antifungal spray assays against the fungus *Fusarium solani* are shown.

In conclusion, the initial substrate of fragin biosynthesis was investigated using isotopically labeled [U-$^{13}C_5$]-L-valine (**4**) and an incorporation of all the ^{13}C atoms of valine into fragin were observed by HRMS measurement. Furthermore, two possible biosynthetic models suggesting an early or late formation of the diazeniumdiolate functional group were proposed. Several biosynthetic intermediates, hypothesized in the case of a diazeniumdiolate late stage formation, were synthesized and used as analytical standards in the analysis of the wild type and mutant strain (Δ*hamA*, Δ*hamB*, Δ*hamC*, Δ*hamD*, Δ*hamE*, Δ*hamF*, and Δ*hamG*) supernatants. Only the oxime **9** was detected in the wild type strain and was identified in a previous report as a degradation product of fragin.[10] These results suggest that the

diazeniumdiolate could be formed during an early stage of the biosynthesis.

Additionally, a SAR study was performed on fragin in relation with its antibacterial and antifungal activity. Derivatives with a modified acyl side chain, a modified amino acid moiety and deactivation of the diazeniumdiolate were synthesized and their activities against the fungus *F. solani* and several Gram-positive and Gram-negative bacteria were evaluated. An eight C atom acyl side chain, an amino acid possessing an isopropyl group and an underivatized diazeniumdiolate were found to be essential to retain a good activity against Gram-positive bacteria. All the tested compounds possessed a weak potency against Gram-negative bacteria and the derivatives with a long acyl side chain or a methylated diazeniumdiolate lost their activity. In contrast to antibacterial activity, we found that even minor structural changes of fragin greatly reduced its antifungal activity.

Experimental Section

The experimental procedures and the characterization of the compounds are found in the supporting information.

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Keywords: diazeniumdiolate • biosynthesis • natural product • fragin • structure and reactivity

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